Host Defense (Antimicrobial) Peptide, Human β-Defensin-3, Improves the Function of the Epithelial Tight-Junction Barrier in Human Keratinocytes

Chanisa Kiatsurayanon[1,2], François Niyonsaba[1], Rithee Smithrithee[1,2], Toshihiro Akiyama[1,2], Hiroko Ushio[1], Mutsuko Hara[1], Ko Okumura[1], Shigaku Ikeda[1,2] and Hideoki Ogawa[1]

Human β-defensins (hBDs) are host defense peptides that not only exhibit microbicidal properties but also stimulate various cellular activities, including keratinocyte proliferation, migration, and wound healing. hBDs are overexpressed in the skin in cases of psoriasis but are downregulated in atopic dermatitis skin, although both diseases are associated with stratum corneum barrier defects. Because the tight-junction (TJ) barrier is also dysfunctional in both atopic dermatitis and psoriasis patients, we hypothesized that hBDs may regulate the TJ barrier function in keratinocytes. We observed that, among the hBDs tested, only hBD-3 increased the expression of several claudins and their localization along the cell–cell borders. In addition, hBD-3 elevated the transepithelial electrical resistance and reduced the paracellular permeability of keratinocyte layers, and this effect was reversed by the claudin inhibitor ochratoxin A, CCR6 antibody, and CCR6 small interfering RNA. Moreover, hBD-3 enhanced the activation of Rac1, atypical protein kinase C, glycogen synthase kinase-3, and phosphatidylinositol 3 kinase, which are required for the hBD-3-mediated regulation of the TJ barrier function, as evidenced by the effects of their respective inhibitors. Collectively, our findings provide evidence regarding the contribution of host defense peptides to the innate immunity of skin by regulating TJ barrier function, in addition to their antimicrobial and other immunomodulatory activities.

Journal of Investigative Dermatology (2014) **134,** 2163–2173; doi:10.1038/jid.2014.143; published online 17 April 2014

INTRODUCTION

The skin upholds homeostasis by preventing water loss via evaporation and precluding the penetration of exogenous substances due to its structural integrity and secretion of complement proteins and cytokines/chemokines (Baroni *et al.*, 2012). As an antimicrobial barrier, the skin generates a number of antimicrobial or host defense peptides (HDPs). Besides their antimicrobial activities, HDPs exhibit a wide variety of immunomodulatory functions, including promoting chemotaxis, stimulating chemokine/cytokine production, suppressing proinflammatory responses, and inducing cell proliferation, angiogenesis, and wound healing (Niyonsaba *et al.*, 2006, 2009).

The major HDPs found in human skin include the human β-defensins (hBDs) and LL-37. The hBDs are characterized by the presence of three family-specific disulfide bonds and are produced mainly by the epithelia of several organs, including the skin (Ganz, 2003; Niyonsaba *et al.*, 2006). Four hBDs, hBD-1 through hBD-4, have been identified in human skin to date. hBD-1 is constitutively expressed by various epithelial tissues, particularly in the skin, urogenital, and respiratory tissues (Fulton *et al.*, 1997; Ali *et al.*, 2001). hBD-2 was initially identified in psoriatic lesions and was later found in keratinocytes (Harder *et al.*, 1997; Liu *et al.*, 2002). hBD-3 was first isolated from lesional scales, and although it is a psoriatic product, it is also abundant in nonepithelial tissues (Harder *et al.*, 2001). Finally, hBD-4 has been identified in the skin only at the mRNA level (Garcia *et al.*, 2001) and is inducible in differentiated keratinocytes (Harder *et al.*, 2004). In the skin, hBDs have been reported to induce keratinocyte migration, proliferation, and cytokine/chemokine production and to accelerate wound healing (Sorensen *et al.*, 2003; Niyonsaba *et al.*, 2005, 2007). Skin-derived HDPs are closely associated with skin diseases. For instance, the expression of hBDs and LL-37 is upregulated in cases of wounding, acne vulgaris, and psoriasis (Sorensen *et al.*, 2003; Niyonsaba *et al.*, 2005, 2007); on the contrary, their expression is downregulated in atopic dermatitis (AD) (Ong *et al.*, 2002; Nomura *et al.*, 2003). This finding explains why patients

[1]*Atopy (Allergy) Research Center, Juntendo University Graduate School of Medicine, Tokyo, Japan and* [2]*Department of Dermatology, Juntendo University Graduate School of Medicine, Tokyo, Japan*

Correspondence: François Niyonsaba, Atopy (Allergy) Research Center, Juntendo University Graduate School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan. E-mail: francois@juntendo.ac.jp

Abbreviations: aPKC, atypical protein kinase C; GSK-3, glycogen synthase kinase-3; hBD, human β-defensin; HDP, host defense peptide; PI3K, phosphatidylinositol 3 kinase; TER, transepithelial electrical resistance; TJ, tight junction

Received 16 June 2013; revised 20 February 2014; accepted 28 February 2014; accepted article preview online 14 March 2014; published online 17 April 2014

with AD but not psoriasis often demonstrate increased susceptibility to infections.

It was recently demonstrated that uninvolved AD skin, in contrast to psoriatic skin, has a markedly reduced content of tight-junction (TJ) proteins, resulting in barrier dysfunction and immune dysregulation in AD patients (De Benedetto *et al.*, 2011). TJs are cell–cell junctions that form the major barrier controlling the paracellular permeability of water, ions, and solutes across epithelial cell sheets (Kirschner *et al.*, 2013). TJs mainly consist of claudins, occludin, junctional adhesion molecules, and the zonula occludens (Niessen, 2007). Claudins are essential for the regulation of TJ function either by enhancing the barrier, e.g., claudin-1, which is crucial for tightening the epithelial barrier (Furuse *et al.*, 2002) or disrupting the barrier, e.g., claudin-2, which attenuates the tightness of the epithelial barrier (Furuse *et al.*, 2001). Although both psoriasis and AD are associated with an impaired stratum corneum barrier, the TJ protein expression is downregulated in AD (De Benedetto *et al.*, 2011), but it is normal, increased, or decreased in psoriasis (Itoh *et al.*, 2005; Peltonen *et al.*, 2007; Kirschner *et al.*, 2009). Because the expression of hBDs is reduced in AD but increased in psoriasis, our objective was to investigate the effects of hBDs on the TJ barrier function in skin and to examine the possible underlying mechanisms of the effects.

Here, we demonstrated that hBD-3 enhanced the expression and cell membrane localization of certain claudins and elevated transepithelial electrical resistance (TER) while reducing paracellular permeability in keratinocyte layers, through CCR6 signaling. In addition, hBD-3 activated the Rac1, atypical atypical protein kinase C (aPKC), glycogen synthase kinase (GSK)-3, and phosphatidylinositol 3 kinase (PI3K) pathways, which are necessary for the hBD-3-mediated regulation of the TJ barrier function. These observations provide evidence of the contribution of HDPs to the innate immunity of the skin through regulating the TJ barrier function.

RESULTS

hBD-3 increases the expression and membrane localization of TJ proteins

Among the four hBDs, only hBD-3 significantly and dose-dependently upregulated (from 4- to 60-fold) the expression of claudin-1 through claudin-5, claudin-9, claudin-11, claudin-14 through claudin-17, claudin-20, claudin-23, and claudin-25 (Figure 1a). In contrast, hBD-3 did not affect the expression of occludin, cadherin, and zonula occludens (data not shown), suggesting that it is a selective effector. Unexpectedly, hBD-1, -2, and -4, even at doses as high as 40 $\mu g\,ml^{-1}$, did not increase the expression of the TJ proteins tested (data not shown). Western blot analysis revealed that only claudin-1, claudin-3, claudin-4, claudin-14, and claudin-23 were enhanced at the protein level following hBD-3 stimulation (Figure 1b). Because the cellular localization of TJ proteins is essential for functional TJ formation, the effect of hBD-3 on cellular distribution of claudins was examined. hBD-3 induced a widespread localization of claudin-1, claudin-3, claudin-4, and claudin-14, and a slight increase of claudin-23 at the cell–cell borders (Figure 2). Thus, the selective effect of hBD-3 on certain claudins implies the importance of these molecules in the hBD-3-mediated regulation of TJ barrier function.

Ochratoxin A blocks hBD-3-mediated changes in the TJ barrier function

To examine whether the hBD-3-mediated changes in claudin localization were associated with the TJ function, we assessed the TJ barrier function parameters, TER, and paracellular permeability of FITC–dextran (Matter and Balda, 2003; Yuki *et al.*, 2007). Upon stimulation with hBD-3, the TER of keratinocyte layers dose- and time-dependently increased. The highest TER value was observed at 72 hours, and it then gradually declined (Figure 3a). Furthermore, as shown in Figure 3b, control keratinocyte layers exposed to the vehicle exhibited high levels of 4 kDa FITC–dextran flux from the apical compartment to the basolateral compartment. However, the paracellular flux of FITC–dextran through layers treated with hBD-3 was significantly decreased. Paracellular flux following a 72-hour treatment was maximally inhibited, and it then progressively increased. This indicates that hBD-3 improves the formation and function of the TJ barrier. Interestingly, hBD-1, hBD-2, and hBD-4 neither markedly affected the membrane localization of claudins (Supplementary Figure S1 online), nor increased TJ barrier function as assessed by TER and paracellular flux (Supplementary Figure S2 online).

Because ochratoxin A is able to induce a decrease in the TJ barrier function by removing claudins (McLaughlin *et al.*, 2004; Lambert *et al.*, 2007), keratinocyte layers were treated with ochratoxin A, and the TER and paracellular flux were assessed. Ochratoxin A treatment caused a reduced TER and elevated flux of FITC–dextran in hBD-3-stimulated keratinocyte layers (Figure 3c). This suggests an association between hBD-3-mediated TJ barrier function and reduced expression of claudins.

hBD-3-induced improvement of TJ barrier function is mediated through CCR6

Because hBD-3 functions through CCR6 activation (Wu *et al.*, 2003; Nagaoka *et al.*, 2008), we tested involvement of CCR6 in the hBD-3-mediated regulation of TJ function by treating keratinocyte layers with CCR6-neutralizing antibody or transfecting cells with CCR6 small interfering RNA to knockdown CCR6 receptor (Supplementary Figure S3 online). As pictured in Figure 4a and b, both CCR6-neutralizing antibody and CCR6 small interfering RNA markedly reduced TER and increased the paracellular permeability to the baseline in hBD-3-stimulated layers, demonstrating involvement of CCR6 in hBD-3-mediated improvement of TJ barrier function.

hBD-3 induces activation of aPKC and Rac1, which are necessary for the TJ barrier function

The aPKC family has two isoforms, aPKCζ and aPKCι/λ, which are expressed in keratinocytes and are associated with TJ proteins (Helfrich *et al.*, 2007). Thus, we investigated the possible role of these kinases in hBD-3-mediated regulation of TJ barrier function. We observed that the phosphorylation of

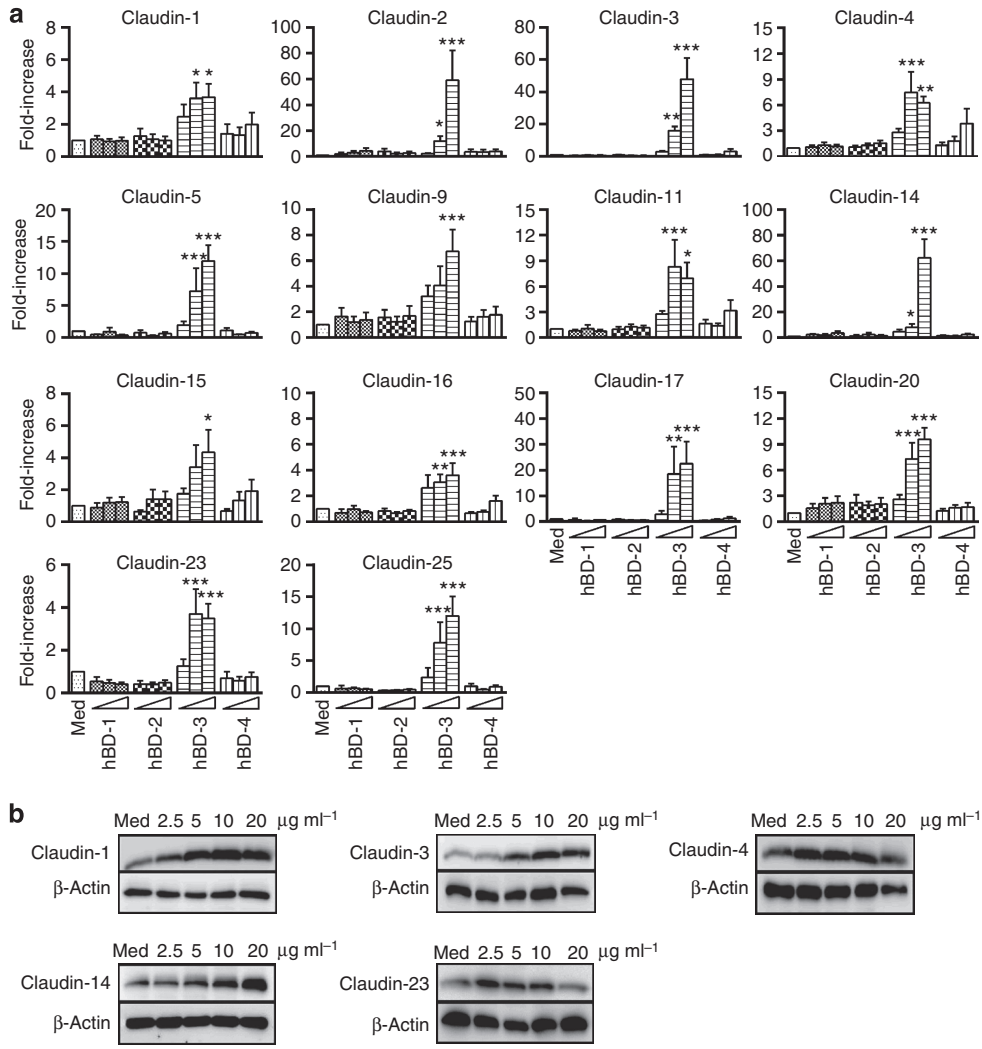

Figure 1. Effects of human β-defensins (hBDs) on the expression of tight-junction (TJ) mRNAs and proteins in keratinocytes. (**a**) Keratinocytes were incubated with 5–20 μg ml^{-1} of hBDs or medium alone for 24–48 hours. Following the incubation, total RNA was extracted and reverse-transcribed into complementary DNA, and quantitative real-time reverse-transcriptase–PCR was performed. The values represent fold-increases in gene expression relative to those of controls stimulated with medium alone (Med). *P<0.05, **P<0.01, ***P<0.001, n=5. (**b**) Keratinocytes were stimulated with 2.5–20 μg ml^{-1} of hBD-3 or the diluent (Med) for 48 hours. The levels of claudin-1, claudin-3, claudin-4, claudin-14, and claudin-23 in cell lysates were determined by western blot. The results of one representative experiment of three separate experiments yielding similar results are shown.

aPKCζ/λ was markedly enhanced in keratinocytes stimulated with hBD-3 for 8 hours (Figure 5a). This activation was necessary for the hBD-3-improved TJ barrier function, because treatment of keratinocyte layers with GF 109203X, a pan-PKC inhibitor that hampers increases in the TER (Turner *et al.*, 1999), notably suppressed hBD-3-induced elevation of the TER and also increased the flux of FITC–dextran (Figure 5b).

Furthermore, as aPKC signaling is activated by guanosine triphosphate (GTP)-bound Rac1-induced protein phosphorylation (Chen and Macara, 2005) and because dominant-negative Rac1 and kinase-dead aPKC inhibit TJ functions (Mertens *et al.*, 2005; Helfrich *et al.*, 2007), we investigated whether hBD-3 regulates TJ barrier function by activating GTP-Rac1. First, Rac1 pull-down assays revealed that hBD-3 stimulation increased the level of activated GTP-Rac1, compared with that of control samples, in as little as 5 minutes, and this effect was sustained for up to 15 minutes (Figure 5c). GTP-Rac1 is indeed required for the TJ barrier function because blockade of Rac1 activation using NSC23766 (Baumer *et al.*, 2009), resulted in reduced TER and enhanced paracellular flux of FITC–dextran in hBD-3-stimulated keratinocyte layers (Figure 5d).

hBD-3 regulates TJ barrier function through activating GSK-3 and PI3K

GSK-3 is implicated in the expression of TJ proteins and the maintenance of the TJ barrier function (Doble and Woodgett, 2003). We therefore hypothesized that hBD-3 might also activate GSK-3 to regulate the TJ barrier function. The results revealed a strong GSK-3α/β (Y279/Y216) signal following a 2-hour stimulation with hBD-3, and GSK-3 activation was

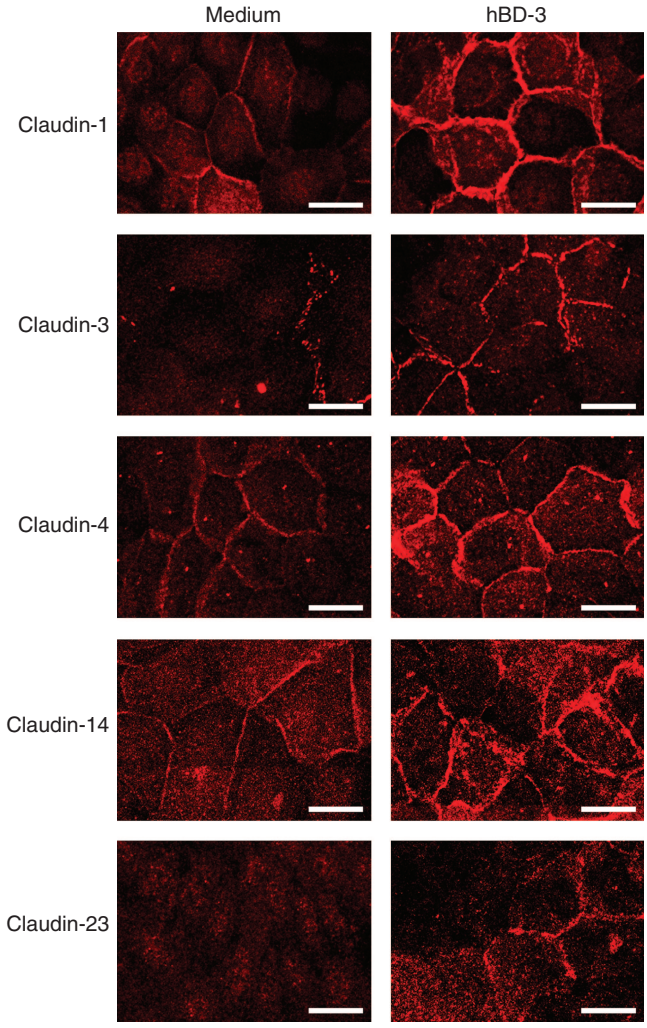

Figure 2. Human β-defensin-3 (hBD-3) enhances immunolocalization of certain claudins. Keratinocytes grown to confluence on collagen I-coated chamber slides were stimulated with 20 μg ml^{-1} hBD-3 for 72 hours. The cells were then fixed in methanol, processed for immunofluorescence with antibodies against claudins overnight, and then visualized using confocal laser scanning microscopy. The results of one representative experiment of three separate experiments yielding similar results are shown. Scale bar = 10 μm.

sustained for up to 8 hours (Figure 6a). This activation was required for the hBD-3-mediated regulation of the TJ barrier function, as confirmed by the effect of the GSK-3 inhibitor, SB 415286 (Severson *et al.*, 2010), that drastically decreased TER, while markedly increasing the paracellular flux (Figure 6b) in hBD-3-stimulated keratinocyte layers.

Moreover, because the PI3K pathway participates in sealing the TJ by improving the TER and TJ protein expression (Gonzalez-Mariscal *et al.*, 2008), we envisaged that hBD-3 might also mediate PI3K activation. Figure 6c shows that during stimulation with hBD-3, the PI3K (p85/p55) signal was strongly increased at 0.5 hours and then gradually decreased. Treatment of keratinocyte layers with wortmannin disrupted the hBD-3-mediated improvement of the barrier function, as demonstrated by the reduced TER and increased paracellular flux (Figure 6d), implying that hBD-3 regulates paracellular permeability through activating PI3K.

DISCUSSION

A recent report that impaired TJ protein expression contributes to the barrier dysfunction in AD (De Benedetto *et al.*, 2011), in contrast to psoriasis, in which the expression of TJ proteins is normal, increased, or decreased (Itoh *et al.*, 2005; Peltonen *et al.*, 2007; Kirschner *et al.*, 2009) incited us to hypothesize that the differential expression of hBDs in psoriasis and AD may have a key role in regulating the TJ barrier function. We demonstrated that hBD-3 selectively increased the expression and membrane localization of certain claudins, enhanced the TER, and reduced paracellular flux in keratinocyte layers. This enhancement of the TJ barrier function was controlled by CCR6, aPKC, GTP-Rac1, GSK-3, and PI3K pathways.

The claudin family provides the most important components of TJs, with more than 24 members that determine TJ resistance and permeability (Van Itallie and Anderson, 2006). We showed that hBD-3 increased the gene expression of claudins, which have been found in keratinocytes (Brandner *et al.*, 2002; Watson *et al.*, 2007; Brandner, 2009; De Benedetto *et al.*, 2011), and demonstrated that keratinocytes also express claudin-20 and claudin-25. Among these claudins, only claudin-1, claudin-3, claudin-4, claudin-14, and claudin-23 were selectively enhanced at the protein level, and their localization at cell–cell borders was also increased, suggesting the importance of these claudins in the TJ barrier function. Our results corroborate previous findings that claudin-1, claudin-4, and claudin-14 improve TJ function (Anderson and Van Itallie, 2009), and that claudin-3 and claudin-23 also have a crucial role in TJ regulation (Wolburg *et al.*, 2003; Milatz *et al.*, 2010; De Benedetto *et al.*, 2011). In contrast, hBD-3 had no effect on the protein expression of claudin-2, claudin-6, and claudin-10 (data not shown), which disrupt the TJ barrier function (Turksen and Troy, 2002; Anderson and Van Itallie, 2009). This suggests that hBD-3 contributes to the regulation of the epidermal barrier function. Notably, the physiological function of the hBD-3-induced upregulation of claudins was confirmed by the increased TER and reduced paracellular flux, and these effects were reversed by a claudin inhibitor.

Besides differential production of HDPs, psoriasis and AD are also characterized by a specific difference in the profile of cytokines/chemokines (Wilsmann-Theis *et al.*, 2008), which are involved in the TJ regulation. For instance, IL-4, IL-13, and eotaxin that are overexpressed in AD attenuate the TJ barrier function (Capaldo and Nusrat, 2009; Jamaluddin *et al.*, 2009), implying that dysregulated TJ function in AD might be a result of these cytokines/chemokines. In contrast, IFN-γ and tumor necrosis factor-α that are overexpressed in psoriasis improve the TJ function (Capaldo and Nusrat, 2009). However, because these cytokines also downregulate the TJ function (Capaldo and Nusrat, 2009), there are still discrepancies in the TJ regulation in psoriasis. Our previously unreported finding proposes that dysregulated hBD-3 expression might play a key role in the TJ defect observed in both AD and psoriasis.

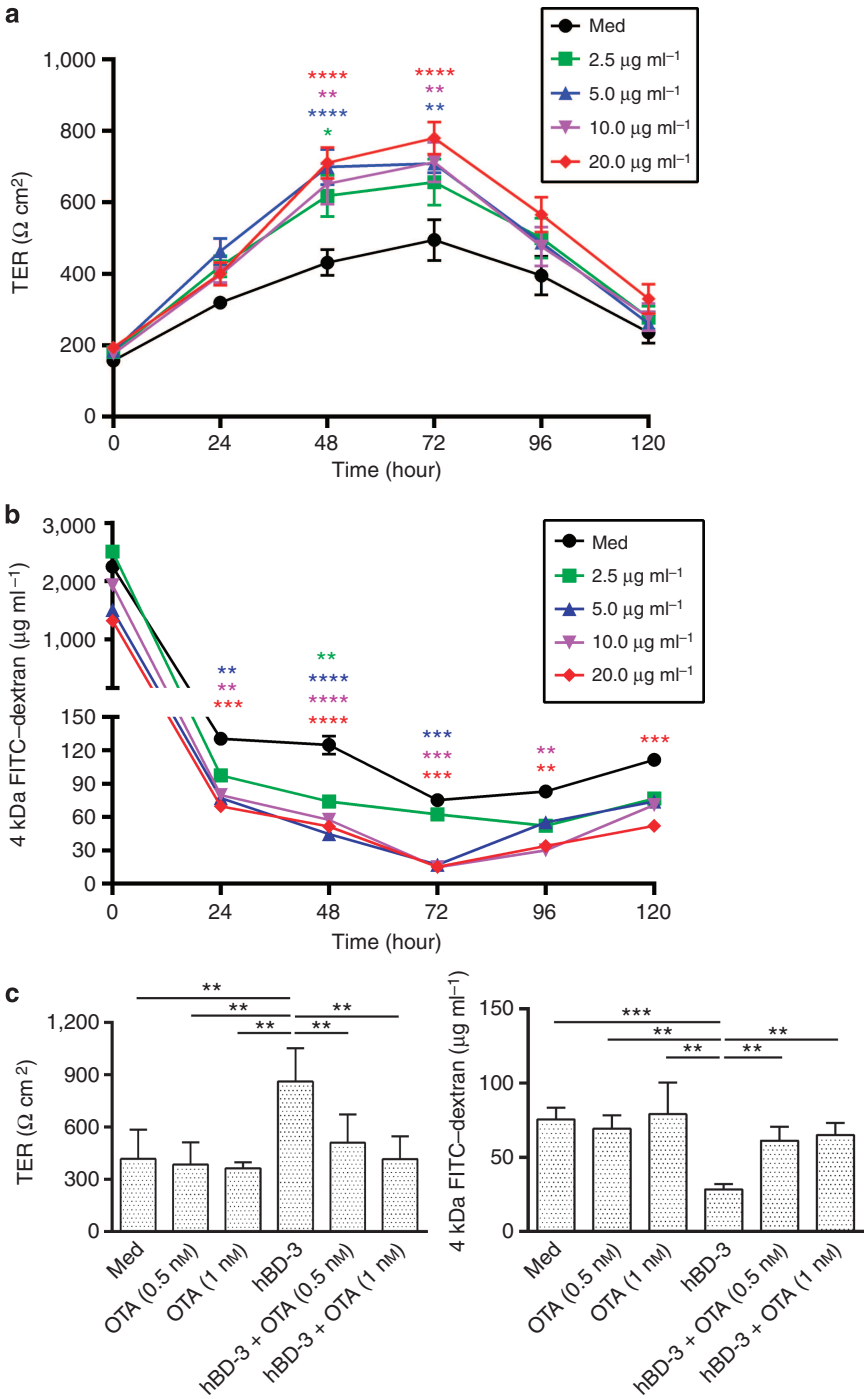

Figure 3. Human β-defensin-3 (hBD-3) enhances the tight-junction (TJ) barrier function. (**a**, **b**) Keratinocyte layers were stimulated with 2.5–20 μg ml^{-1} hBD-3, and the transepithelial electrical resistance (TER) and paracellular flux were determined as described in Materials and Methods. The values obtained using stimulated and nonstimulated cells were compared (Med). *$P<0.05$, **$P<0.01$, ***$P<0.001$, ****$P<0.0001$, $n=5$. (**c**) Keratinocyte layers were pre-treated with 0.5 and 1 nM ochratoxin A (OTA) or 0.1% DMSO for 24 hours, stimulated with 20 μg ml^{-1} hBD-3 or diluent for 72 hours, and then the TER and the paracellular flux were measured. The values obtained using stimulated and nonstimulated cells (Med, OTA) or with the presence and absence of inhibitor were compared. **$P<0.01$, ***$P<0.001$, $n=5$.

Although hBD-1 through hBD-4 activate keratinocytes, we unexpectedly found that only hBD-3 regulates the TJ barrier function. One of the reasonable interpretations for this observation is the possibility that hBD-3 may interact with certain specific proteins in keratinocytes, which other hBDs cannot bind. For instance, although hBDs induce cell

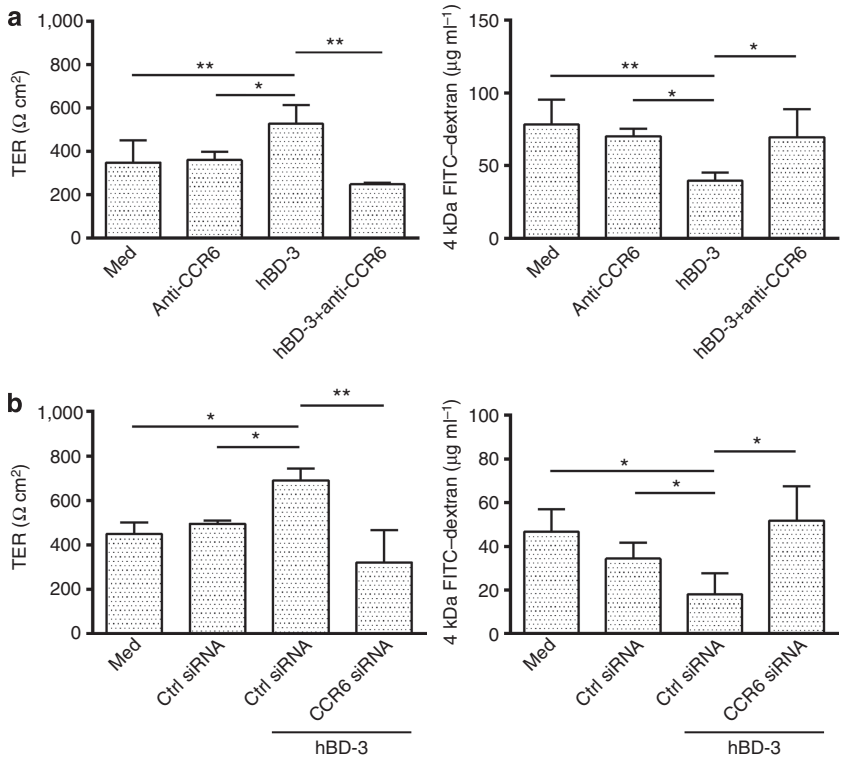

Figure 4. Human β-defensin-3 (hBD-3) enhances the tight-junction (TJ) barrier function through CCR6. (**a**) Keratinocyte layers were pre-treated with 50 μg ml^{-1} anti-CCR6 antibody for 24 hours, stimulated with 20 μg ml^{-1} hBD-3 for 72 hours, and then the transepithelial electrical resistance (TER) and the paracellular flux were measured. *P<0.05, **P<0.01, n=5, compared with the nonstimulated cells, and in the presence and absence of CCR6 antibody. (**b**) Keratinocyte layers were transfected with 400 nM CCR6 small interfering RNA (siRNA) or control siRNA for 48 hours, stimulated with 20 μg ml^{-1} hBD-3 for 72 hours, and the TER and the paracellular flux were measured. *P<0.05, **P<0.01, n=5, compared with the nonstimulated cells, and between CCR6 siRNA-transfected cells and control siRNA-transfected cells.

migration through CCR2, CCR6, and EGFR (Wu *et al.*, 2003; Niyonsaba *et al.*, 2007; Rohrl *et al.*, 2010), only hBD-3 but not other hBDs inhibits neutrophil apoptosis via CCR6 (Nagaoka *et al.*, 2008). This incited us to explore the role of CCR6 in hBD-3-mediated TJ barrier function regulation. Neutralizing and knocking down CCR6 suppressed hBD-3-induced improvement of TJ barrier function, confirming the requirement of this receptor in hBD-3 regulation of TJ function. However, because hBD-3 is a very strong cationic peptide (with a net charge of +11) compared with other hBDs, one could not exclude the possibility that hBD-3 might also efficiently act upon nonselective membrane receptors to activate keratinocytes, as reported for many cationic peptides (Ferry *et al.*, 2002).

To further investigate the molecular mechanisms by which hBD-3 increases the TJ barrier function, we focused on aPKC, Rac1, GSK-3, and PI3K that regulate TJ formation (Mertens *et al.*, 2005; Helfrich *et al.*, 2007; Gonzalez-Mariscal *et al.*, 2008; Severson *et al.*, 2010). The overexpression of aPKC is sufficient for TJ formation in keratinocytes in which TJ formation is defective, and TJ formation is regulated by the polarity protein complex Par3/Par6/aPKC that functions downstream of Rac1 (Mertens *et al.*, 2005). Furthermore, low GTP-Rac1 levels and reduced aPKC activity suppress the maturation of TJs (Mertens *et al.*, 2005), and UVB-irradiation of skin causes the deterioration of TJ functionality, which is accompanied by the downregulation of GTP-Rac1 and aPKC (Yuki *et al.*, 2011).

Recently, the activity of GSK-3 has also been implicated in regulating the TJ barrier function. Although GSK-3 was generally considered a constitutive active enzyme, only regulated by inhibition through its phosphorylation at S21/S9 (Doble and Woodgett, 2003), recent reports have indicated that phosphorylation of GSK-α/β at Y279/Y216 leads to GSK-3 activation (Bhat *et al.*, 2000; Sayas *et al.*, 2002). Therefore, our findings that hBD-3 increased the phosphorylation of GSK-α/β at Y279/Y216 but not S21/S9 (data not shown), and that the inhibition of GSK-3 activity reversed TJ barrier function suggested that hBD-3 improves TJ function through GSK-3 activation. This is consistent with studies showing that inhibiting GSK-α/β increases epithelial permeability, and this is attributable to the downregulation of TJ protein expression (Bachelder *et al.*, 2005; Severson *et al.*, 2010).

Because of evidence implicating PI3K in TJ assembly, the role of the PI3K signaling pathway in the hBD-3-mediated regulation of the TJ barrier function was studied. Our finding is supported by a study showing the requirement for PI3K in Der p2-induced claudin-2 expression in lung alveolar cells

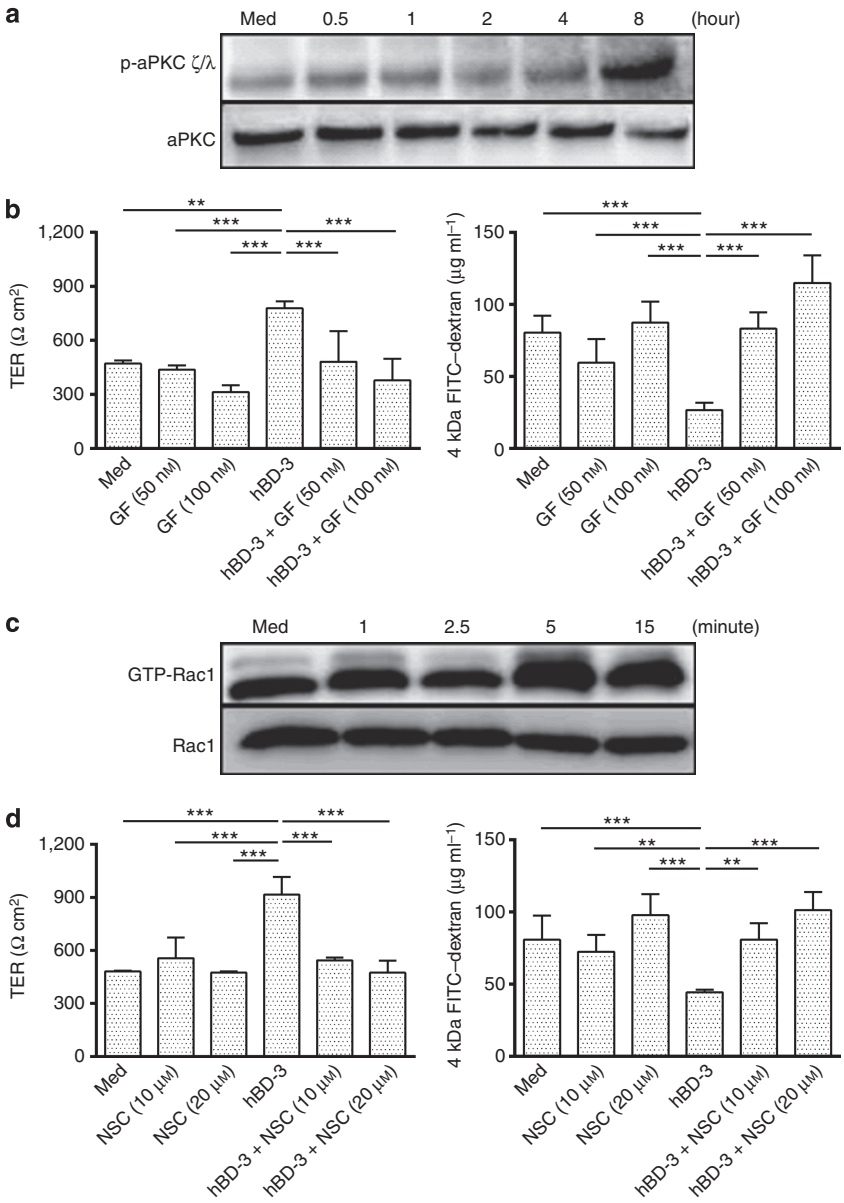

Figure 5. Human β-defensin-3 (hBD-3) enhances the tight-junction (TJ) function through activation of atypical protein kinase C (aPKC)ζ/λ and guanosine triphosphate (GTP)-Rac1. Keratinocytes were incubated with 20 μg ml^{-1} hBD-3, and lysates were separated by SDS-PAGE using antibodies against phosphorylated aPKCζ/λ and aPKC (**a**), or quantified for GTP-Rac1 using a Rac1 activation assay kit (**c**). One representative experiment of three separate experiments yielding similar results is shown. Keratinocyte layers were pre-treated with GF 109203X (**b**), NSC23766 (**d**), or 0.1% DMSO for 48 hours and stimulated with 20 μg ml^{-1} hBD-3 for 72 hours, and the transepithelial electrical resistance (TER) and paracellular flux were determined. The values obtained using stimulated and nonstimulated cells or with the presence and absence of inhibitor were compared. *$P<0.05$, **$P<0.01$, ***$P<0.001$, $n=4$.

(Wang *et al.*, 2011) and a report that the PI3K inhibitors attenuate the TER in response to prostaglandins (Little *et al.*, 2003). In addition, as the p85 subunit of PI3K binds to occludin, this further suggests the importance of PI3K in TJ tightening (Nusrat *et al.*, 2000). However, the activation of the PI3K cascade may also result in opposite effects on the TJ barrier function, as PI3K is implicated in reduced TJ protein expression upon treatment with HIV-1 Tat protein (Andras *et al.*, 2005) or TGFβ1 (Bakin *et al.*, 2000). Hence, further studies are needed to fully clarify the role of the PI3K signaling pathway in the TJ barrier function. Although other hBDs did not affect keratinocyte layer permeability, they were able to induce at some extent the activation of aPKC, Rac1, GSK-3, and PI3K (Supplementary Figure S4 online). It seems that the activation of above pathways by hBD-1, hBD-2, or hBD-4 does not affect the regulation of TJ function, but rather may be involved in other activities such as cell survival, wound healing, migration, or differentiation (Koivisto *et al.*, 2003; Efimova *et al.*, 2004; Calautti *et al.*, 2005; Tscharntke *et al.*, 2007), which are regulated by hBDs (Niyonsaba *et al.*, 2009).

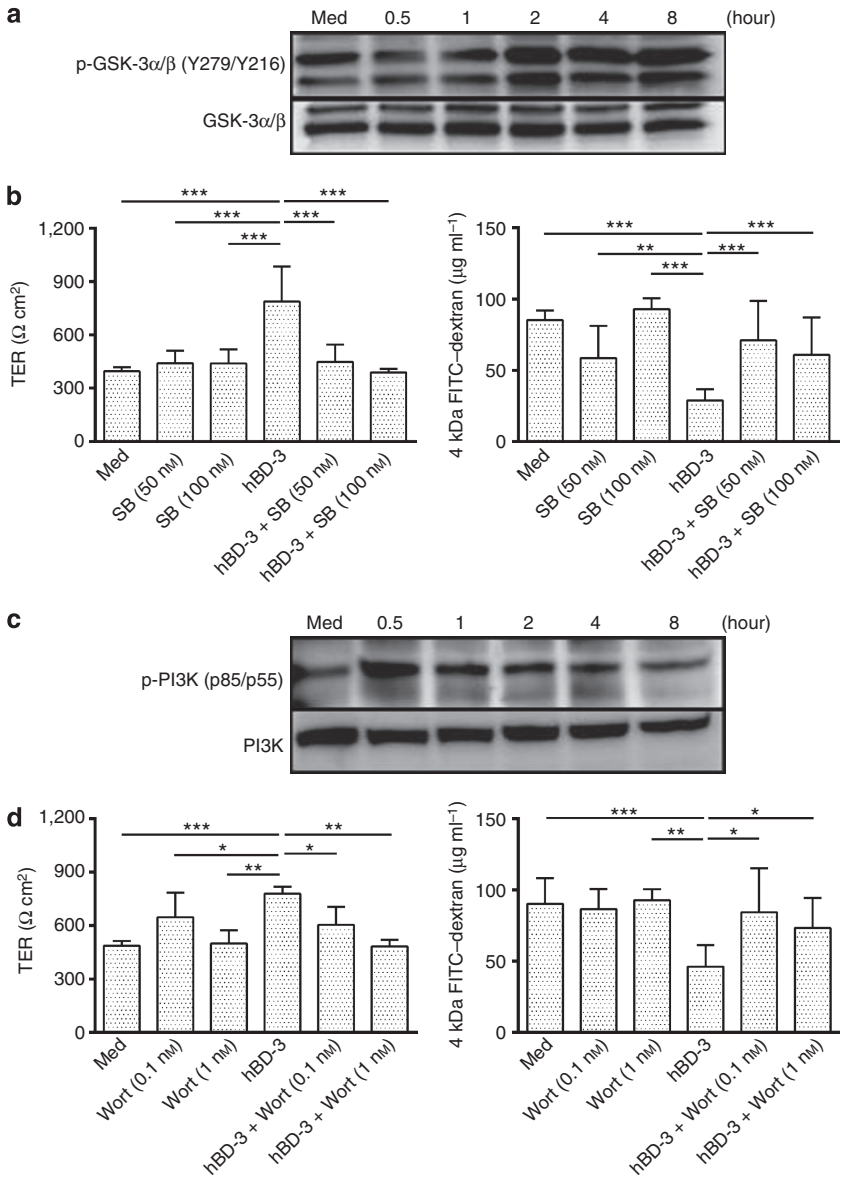

Figure 6. Human β-defensin-3 (hBD-3) enhances the tight-junction (TJ) function through glycogen synthase kinase-3 (GSK-3)α/β and phosphatidylinositol 3 kinase (PI3K) activation. Keratinocytes were incubated with 20 μg ml^{-1} hBD-3, and lysates were separated by SDS-PAGE using antibodies against phosphorylated GSK-3α/β (Y279/Y216) and GSK-3α/β (**a**), or phosphorylated PI3K (p85/p55) and PI3K (**c**). The results of one representative experiment of the three separate experiments yielding similar results are shown. Keratinocyte layers were pre-treated with SB 415286 (SB) (**b**), wortmannin (Wort) (**d**), or 0.1% DMSO for 48 hours and stimulated with 20 μg ml^{-1} hBD-3 for 72 hours, and the transepithelial electrical resistance (TER) and paracellular flux were determined. The values obtained using stimulated and nonstimulated cells or with the presence and absence of inhibitor were compared. *$P<0.05$, **$P<0.01$, ***$P<0.001$, $n=5$.

Other possible TJ function regulators, including Toll-like receptors, protein kinase A, and mitogen-activated protein kinases, were also investigated, but failed to show that hBD-3 regulates the TJ barrier function through these components (data not shown).

Collectively, we demonstrated that hBD-3 enhanced the expression and cellular localization of selective TJ proteins, and regulated the TJ barrier function. Therefore, in addition to their antimicrobial and immunomodulatory functions, we provide previously unreported evidence that HDPs also contribute to the regulation of the skin barrier function.

MATERIALS AND METHODS

Reagents

Synthetic hBD-1 through hBD-4 were purchased from the Peptide Institute (Osaka, Japan). Anti-claudin-1, claudin-3, and claudin-4 Abs were from Invitrogen (Carlsbad, CA), whereas the anti-claudin-14 and claudin-23 Abs were obtained from Abcam (Tokyo, Japan). Anti-phosphorylated GSK-3α/β (Y279/Y216), aPKCζ/λ, and PI3K Abs, and GSK-3α/β, aPKC, and PI3K Abs were from Cell Signaling Technology (Beverly, MA). Anti-CCR6 antibody was from R&D Systems (Minneapolis, MN). GF 109203X was purchased from Enzo Life Sciences (Farmingdale, NY) and SB 415286 was from Tocris

Bioscience (Bristol, UK), whereas wortmannin and ochratoxin A were obtained from Sigma-Aldrich (St Louis, MO). NSC23766 was purchased from Calbiochem (La Jolla, CA).

Keratinocyte culture and stimulation

Keratinocytes purchased from Kurabo Industries (Osaka, Japan) were cultured in HuMedia-KG2 (Kurabo Industries), as described previously (Niyonsaba *et al.*, 2007). For total RNA extractions and western blotting, the keratinocytes were cultured in 12-well plates and incubated with peptides in HuMedia without supplements. For the TER and paracellular flux assays, 0.72×10^5 cells were plated in transwells with 0.4-μm pores (Millipore, Billerica, MA), and the medium was replaced with high-Ca^{2+} (1.35 mM) medium after the cells reached confluence.

Total RNA extraction and quantitative real-time reverse-transcriptase–PCR

Total RNA extractions and quantitative real-time reverse-transcriptase–PCR were performed as previously described (Niyonsaba *et al.*, 2007). mRNAs were amplified and quantitated using a StepOne Plus Real-time PCR System (Applied Biosystems, Branchburg, NJ), following the manufacturer's instructions. The primer/probe sets used were Applied Biosystems assays-on-demand products. The changes in gene expression are reported as fold-increases relative to those of untreated controls.

Western blot analysis

Keratinocytes were incubated with hBDs for the indicated periods. After stimulation, the cells were lysed in radioimmunoprecipitation assay buffer (Cell Signaling Technology) and equal amounts of lysate proteins were subjected to 12.5% SDS-PAGE. The immunoblots were incubated with appropriate Abs according to the manufacturer's instructions. The membranes were developed using Luminata Forte Western HRP substrate (Millipore, Billerica, MA).

Measurements of TER and paracellular flux

Keratinocytes grown on 0.6-cm^2 transwell filters were transferred into the high-Ca^{2+} medium, and hBDs were added. The TER across the keratinocyte layers was measured using a CellZscope (nanoAnalytics, Münster, Germany) for 0–120 hours. Keratinocytes were also grown on 0.3-cm^2 transwell filters for the paracellular flux assay, which was performed as described previously (Yuki *et al.*, 2007), using 4 kDa FITC–dextran (Sigma-Aldrich) as a tracer. Briefly, keratinocyte layers were stimulated with hBDs for 0–120 hours, and the medium in the apical and basal compartments was replaced with 250 μl of P buffer containing 10 mg ml^{-1} of FITC–dextran and 700 μl of P buffer, respectively. After 2 hours, the medium from the basal compartment was collected and fluorescence was measured using a fluorimeter (Nihon Molecular Devices, Tokyo, Japan). In some of the experiments, the keratinocyte layers were pre-treated with specific inhibitors or Abs, or transfected with CCR6 small interfering RNA (Invitrogen) before stimulation, and the efficacy of knockdown was confirmed by quantitative real-time reverse-transcriptase–PCR (Supplementary Figure S3 online). Inhibitors used in this study had no cytotoxicity as evaluated by trypan blue exclusion and lactate dehydrogenase activity (data not shown). Following treatment, keratinocyte layers were stimulated, and the TER and paracellular flux assays were performed as above.

Rac1 activation assay

Keratinocytes grown in 12-well plates were stimulated with hBDs for 1–15 minutes. The cells were lysed in radioimmunoprecipitation assay buffer supplemented with protease inhibitor cocktail, and Rac1 activation in the lysates was determined using a Rac1 activation assay kit (Cytoskeleton, Denver, CO), according to the manufacturer's recommendations.

Immunofluorescence microscopy

Keratinocytes were cultured on collagen I-coated chamber slides (BD Biosciences, Bedford, MA) before fixation with methanol. Cells were blocked in Protein Block Serum-Free (DakoCytomation, Carpentaria, CA) containing 0.2% Tween-20, after which cells were incubated overnight with the appropriate primary Abs in 1% BSA–PBS containing 0.2% Tween-20, followed by specific secondary Abs coupled to Alexa 594 (Invitrogen). Images were captured using confocal laser scanning microscopy (Carl Zeiss, Jena, Germany).

Statistical analysis

Statistical analysis was performed with an analysis of variance using Prism GraphPad software (GraphPad Software, San Diego, CA). $P<0.05$ was considered significant. The results are presented as the mean values ± SD.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

We would like to thank all of the members of the Atopy (Allergy) Research Center and the Department of Immunology of the Juntendo University Graduate School of Medicine for their comments and encouragement, and Michiyo Matsumoto for secretarial assistance. This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan to the Atopy (Allergy) Research Center, Juntendo University, Tokyo, Japan.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

REFERENCES

Ali RS, Falconer A, Ikram M *et al.* (2001) Expression of the peptide antibiotics human beta defensin-1 and human beta defensin-2 in normal human skin. *J Invest Dermatol* 117:106–11

Anderson JM, Van Itallie CM (2009) Physiology and function of the tight junction. *Cold Spring Harb Perspect Biol* 1:a002584

Andras IE, Pu H, Tian J *et al.* (2005) Signaling mechanisms of HIV-1 Tat-induced alterations of claudin-5 expression in brain endothelial cells. *J Cereb Blood Flow Metab* 25:1159–70

Bachelder RE, Yoon SO, Franci C *et al.* (2005) Glycogen synthase kinase-3 is an endogenous inhibitor of Snail transcription: implications for the epithelial-mesenchymal transition. *J Cell Biol* 168:29–33

Bakin AV, Tomlinson AK, Bhowmick NA *et al.* (2000) Phosphatidylinositol 3-kinase function is required for transforming growth factor beta-mediated epithelial to mesenchymal transition and cell migration. *J Biol Chem* 275:36803–10

Baroni A, Buommino E, De Gregorio V *et al.* (2012) Structure and function of the epidermis related to barrier properties. *Clin Dermatol* 30:257–62

Baumer Y, Spindler V, Werthmann RC *et al.* (2009) Role of Rac 1 and cAMP in endothelial barrier stabilization and thrombin-induced barrier breakdown. *J Cell Physiol* 220:716–26

Bhat RV, Shanley J, Correll MP *et al.* (2000) Regulation and localization of tyrosine216 phosphorylation of glycogen synthase kinase-3beta in cellular and animal models of neuronal degeneration. *Proc Natl Acad Sci USA* 97:11074–9

Brandner JM (2009) Tight junctions and tight junction proteins in mammalian epidermis. *Eur J Pharm Biopharm* 72:289–94

Brandner JM, Kief S, Grund C *et al.* (2002) Organization and formation of the tight junction system in human epidermis and cultured keratinocytes. *Eur J Cell Biol* 81:253–63

Calautti E, Li J, Saoncella S *et al.* (2005) Phosphoinositide 3-kinase signaling to Akt promotes keratinocyte differentiation versus death. *J Biol Chem* 280: 32856–65

Capaldo CT, Nusrat A (2009) Cytokine regulation of tight junctions. *Biochim Biophys Acta* 1788:864–71

Chen X, Macara IG (2005) Par-3 controls tight junction assembly through the Rac exchange factor Tiam1. *Nat Cell Biol* 7:262–9

De Benedetto A, Rafaels NM, McGirt LY *et al.* (2011) Tight junction defects in patients with atopic dermatitis. *J Allergy Clin Immunol* 127:e1–7

Doble BW, Woodgett JR (2003) GSK-3: tricks of the trade for a multi-tasking kinase. *J Cell Sci* 116:1175–86

Efimova T, Broome AM, Eckert RL (2004) Protein kinase Cdelta regulates keratinocyte death and survival by regulating activity and subcellular localization of a p38delta-extracellular signal-regulated kinase 1/2 complex. *Mol Cell Biol* 24:8167–83

Ferry X, Brehin S, Kamel R *et al.* (2002) G protein-dependent activation of mast cell by peptides and basic secretagogues. *Peptides* 23:1507–15

Fulton C, Anderson GM, Zasloff M *et al.* (1997) Expression of natural peptide antibiotics in human skin. *Lancet* 350:1750–1

Furuse M, Furuse K, Sasaki H *et al.* (2001) Conversion of zonulae occludentes from tight to leaky strand type by introducing claudin-2 into Madin-Darby canine kidney I cells. *J Cell Biol* 153:263–72

Furuse M, Hata M, Furuse K *et al.* (2002) Claudin-based tight junctions are crucial for the mammalian epidermal barrier: a lesson from claudin-1-deficient mice. *J Cell Biol* 156:1099–111

Ganz T (2003) Defensins: antimicrobial peptides of innate immunity. *Nat Rev Immunol* 3:710–20

Garcia JR, Krause A, Schulz S *et al.* (2001) Human beta-defensin 4: a novel inducible peptide with a specific salt-sensitive spectrum of antimicrobial activity. *FASEB J* 15:1819–21

Gonzalez-Mariscal L, Tapia R, Chamorro D (2008) Crosstalk of tight junction components with signaling pathways. *Biochim Biophys Acta* 1778: 729–56

Harder J, Bartels J, Christophers E *et al.* (1997) A peptide antibiotic from human skin. *Nature* 387:861

Harder J, Bartels J, Christophers E *et al.* (2001) Isolation and characterization of human beta -defensin-3, a novel human inducible peptide antibiotic. *J Biol Chem* 276:5707–13

Harder J, Meyer-Hoffert U, Wehkamp K *et al.* (2004) Differential gene induction of human beta-defensins (hBD-1, -2, -3, and -4) in keratinocytes is inhibited by retinoic acid. *J Invest Dermatol* 123:522–9

Helfrich I, Schmitz A, Zigrino P *et al.* (2007) Role of aPKC isoforms and their binding partners Par3 and Par6 in epidermal barrier formation. *J Invest Dermatol* 127:782–91

Itoh K, Kawasaki S, Kawamoto S *et al.* (2005) Identification of differentially expressed genes in psoriasis using expression profiling approaches. *Exp Dermatol* 14:667–74

Jamaluddin MS, Wang X, Wang H *et al.* (2009) Eotaxin increases monolayer permeability of human coronary artery endothelial cells. *Arterioscler Thromb Vasc Biol* 29:2146–52

Kirschner N, Poetzl C, von den Driesch P *et al.* (2009) Alteration of tight junction proteins is an early event in psoriasis: putative involvement of proinflammatory cytokines. *Am J Pathol* 175:1095–106

Kirschner N, Rosenthal R, Furuse M *et al.* (2013) Contribution of tight junction proteins to ion, macromolecule, and water barrier in keratinocytes. *J Invest Dermatol* 133:1161–9

Koivisto L, Alavian K, Hakkinen L *et al.* (2003) Glycogen synthase kinase-3 regulates formation of long lamellipodia in human keratinocytes. *J Cell Sci* 116:3749–60

Lambert D, Padfield PJ, McLaughlin J *et al.* (2007) Ochratoxin A displaces claudins from detergent resistant membrane microdomains. *Biochem Biophys Res Commun* 358:632–6

Little D, Dean RA, Young KM *et al.* (2003) PI3K signaling is required for prostaglandin-induced mucosal recovery in ischemia-injured porcine ileum. *Am J Physiol Gastrointest Liver Physiol* 284:G46–56

Liu AY, Destoumieux D, Wong AV *et al.* (2002) Human beta-defensin-2 production in keratinocytes is regulated by interleukin-1, bacteria, and the state of differentiation. *J Invest Dermatol* 118:275–81

Matter K, Balda MS (2003) Functional analysis of tight junctions. *Methods* 30:228–34

McLaughlin J, Padfield PJ, Burt JP *et al.* (2004) Ochratoxin A increases permeability through tight junctions by removal of specific claudin isoforms. *Am J Physiol Cell Physiol* 287:C1412–7

Mertens AE, Rygiel TP, Olivo C *et al.* (2005) The Rac activator Tiam1 controls tight junction biogenesis in keratinocytes through binding to and activation of the Par polarity complex. *J Cell Biol* 170:1029–37

Milatz S, Krug SM, Rosenthal R *et al.* (2010) Claudin-3 acts as a sealing component of the tight junction for ions of either charge and uncharged solutes. *Biochim Biophys Acta* 1798:2048–57

Nagaoka I, Niyonsaba F, Tsutsumi-Ishii Y *et al.* (2008) Evaluation of the effect of human beta-defensins on neutrophil apoptosis. *Int Immunol* 20:543–53

Niessen CM (2007) Tight junctions/adherens junctions: basic structure and function. *J Invest Dermatol* 127:2525–32

Niyonsaba F, Nagaoka I, Ogawa H (2006) Human defensins and cathelicidins in the skin: beyond direct antimicrobial properties. *Crit Rev Immunol* 26:545–76

Niyonsaba F, Nagaoka I, Ogawa H *et al.* (2009) Multifunctional antimicrobial proteins and peptides: natural activators of immune systems. *Curr Pharm Des* 15:2393–413

Niyonsaba F, Ushio H, Nagaoka I *et al.* (2005) The human beta-defensins (-1, -2, -3, -4) and cathelicidin LL-37 induce IL-18 secretion through p38 and ERK MAPK activation in primary human keratinocytes. *J Immunol* 175:1776–84

Niyonsaba F, Ushio H, Nakano N *et al.* (2007) Antimicrobial peptides human beta-defensins stimulate epidermal keratinocyte migration, proliferation and production of proinflammatory cytokines and chemokines. *J Invest Dermatol* 127:594–604

Nomura I, Goleva E, Howell MD *et al.* (2003) Cytokine milieu of atopic dermatitis, as compared to psoriasis, skin prevents induction of innate immune response genes. *J Immunol* 171:3262–9

Nusrat A, Chen JA, Foley CS *et al.* (2000) The coiled-coil domain of occludin can act to organize structural and functional elements of the epithelial tight junction. *J Biol Chem* 275:29816–22

Ong PY, Ohtake T, Brandt C *et al.* (2002) Endogenous antimicrobial peptides and skin infections in atopic dermatitis. *N Engl J Med* 347:1151–60

Peltonen S, Riehokainen J, Pummi K *et al.* (2007) Tight junction components occludin, ZO-1, and claudin-1, -4 and -5 in active and healing psoriasis. *Br J Dermatol* 156:466–72

Rohrl J, Yang D, Oppenheim JJ *et al.* (2010) Human beta-defensin 2 and 3 and their mouse orthologs induce chemotaxis through interaction with CCR2. *J Immunol* 184:6688–94

Sayas CL, Avila J, Wandosell F (2002) Glycogen synthase kinase-3 is activated in neuronal cells by Galpha12 and Galpha13 by Rho-independent and Rho-dependent mechanisms. *J Neurosci* 22:6863–75

Severson EA, Kwon M, Hilgarth RS *et al.* (2010) Glycogen Synthase Kinase 3 (GSK-3) influences epithelial barrier function by regulating occludin, claudin-1 and E-cadherin expression. *Biochem Biophys Res Commun* 397:592–7

Sorensen OE, Cowland JB, Theilgaard-Monch K *et al.* (2003) Wound healing and expression of antimicrobial peptides/polypeptides in human keratinocytes, a consequence of common growth factors. *J Immunol* 170:5583–9

Tscharntke M, Pofahl R, Chrostek-Grashoff A *et al.* (2007) Impaired epidermal wound healing *in vivo* upon inhibition or deletion of Rac1. *J Cell Sci* 120:1480–90

Turksen K, Troy TC (2002) Permeability barrier dysfunction in transgenic mice overexpressing claudin 6. *Development* 129:1775–84

Turner JR, Angle JM, Black ED *et al.* (1999) PKC-dependent regulation of transepithelial resistance: roles of MLC and MLC kinase. *Am J Physiol* 277:C554–62

Van Itallie CM, Anderson JM (2006) Claudins and epithelial paracellular transport. *Annu Rev Physiol* 68:403–29

Wang WC, Tsai JJ, Kuo CY *et al.* (2011) Non-proteolytic house dust mite allergen, Der p 2, upregulated expression of tight junction molecule claudin-2 associated with Akt/GSK-3beta/beta-catenin signaling pathway. *J Cell Biochem* 112:1544–51

Watson RE, Poddar R, Walker JM *et al.* (2007) Altered claudin expression is a feature of chronic plaque psoriasis. *J Pathol* 212:450–8

Wilsmann-Theis D, Hagemann T, Jordan J *et al.* (2008) Facing psoriasis and atopic dermatitis: are there more similarities or more differences? *Eur J Dermatol* 18:172–80

Wolburg H, Wolburg-Buchholz K, Kraus J *et al.* (2003) Localization of claudin-3 in tight junctions of the blood-brain barrier is selectively lost during experimental autoimmune encephalomyelitis and human glioblastoma multiforme. *Acta Neuropathol* 105:586–92

Wu Z, Hoover DM, Yang D *et al.* (2003) Engineering disulfide bridges to dissect antimicrobial and chemotactic activities of human beta-defensin 3. *Proc Natl Acad Sci USA* 100:8880–5

Yuki T, Hachiya A, Kusaka A *et al.* (2011) Characterization of tight junctions and their disruption by UVB in human epidermis and cultured keratinocytes. *J Invest Dermatol* 131:744–52

Yuki T, Haratake A, Koishikawa H *et al.* (2007) Tight junction proteins in keratinocytes: localization and contribution to barrier function. *Exp Dermatol* 16:324–30